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Positive interference of silicon on water relations, nitrogen metabolism, and osmotic adjustment in two pepper (*Capsicum annuum*) cultivars under water deficit

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Abstract

Silicon actuation can influence the physiological parameters and nitrogen metabolism of pepper. The aim of this study was to (i) investigate the silicon action on nitrogen metabolism and (ii) elucidate the mechanism responsible for osmotic adjustment in two *Capsicum annuum* cultivars with high commercial values exposed to water deficiency. Experimental design used was a completely randomised factorial layout composed of five water and Si treatment combinations (control, deficit + 0.00, deficit + 0.25, deficit + 1.00, and deficit + 1.75 µM Si), applied to two cultivars (Ikeda and Vermelho Gigante) with six replicates. Parameters evaluated were leaf relative water content, stomatal conductance, transpiration rate, nitrate reductase activity, free ammonium, total soluble amino acids, total soluble proteins, proline and glycinebetaine. Proline levels in both cultivars were increased with silicon application in 0.25 µM Si, if compared with the control and deficit + 0 µM Si. A higher glycinebetaine concentration was found in Ikeda cv. at 0.25 µM Si, when compared to control and deficit + 0 µM Si. The data suggests positive inference of silicon on water relations and nitrogen compounds, and an improvement in osmotic adjustment in *Capsicum annuum* plants exposed to water deficit. Proline and glycinebetaine contribute to osmotic adjustment in Ikeda, while this process in Vermelho Gigante is carried only by glycinebetaine.

Keywords: *Capsicum annuum L.*, pepper, silicon, water deficiency, glycinebetaine.
Abbreviations: Si - silicon, $Si(OH)_4$ - monosilicic acid, µM - micromol, CO_2 - carbon dioxide, ATP - Adenosine Triphosphate, H_2O - water, NO_3^- - nitrate, NO_2^- - nitrite, NH_4^+ - ammonium, cv - cultivar, KH_2PO_4 - potassium diphosphate, $(NH_4)_2SO_4$ - ammonium sulphate, NR - nitrate reductase, RiN - nitrite reductase, GS - glutamine synthetase, GOGAT - synthase glutamate, LEA - late embryogenesis abundant.

Introduction

The silicon (Si) is an abundant element in terrestrial superficie (Pereira et al., 2003); however, its availability to plants is normally low (Hattori et al., 2005). According to Matichenkov and Calvert (2002), the chemically active Si in soil is represented by soluble monosilicic acid ($Si(OH)_4$) and acid polisilicic which are soluble and weakly adsorbed, and compound organosilicates, respectively. Silicon is considered a beneficial element to higher plants (Epstein and Bloom, 2004). Its absorption and deposition in cell walls of several organs such as leaf and stem can promote beneficial effects (Cunha et al., 2008). For this reason, it has been frequently linked to physiological, morphological, nutritional, and molecular aspects in plants (Ma, 2004; Epstein, 2004; Ma and Yamaji, 2006; Lobato et al., 2009a).

Pepper (*Capsicum annuum* L.) is a plant used in preparation of foods, condiments, and sauces, being consumed fresh or dehydrated (Sousa et al., 2009). In addition, pepper fruits contain high levels of A and C vitamins (Carvalho et al., 2011). Recent studies reveal that the *Capsicum* genus also has medical properties linked to anti-inflammatory characteristics (Barbosa et al., 2002). On worldwide scale, pepper culture has greater economic importance as it is widely used by the population of several countries. The world yield estimate consists of two types of products, in which China is the largest producer of fresh pepper about 14.5 million tons, and India the main producer of dehydrated pepper in form of condiment at about 1.1 million tons (FAO, 2009). In relation to productive performance of pepper plants, water deficit is a key a limiting factor to achieving adequate yield in protected cultivation or under field conditions (Patanè and Cosentino, 2010).

The stress is defined as a significant change of optimal conditions, which will induce modifications with consequent response in plant metabolism (Lobato et al., 2008a). These changes are reversible or irreversible depending to intensity and duration (Larcher, 2006). Additionally, water deficit is a condition of stress to plants (Costa et al., 2011), and it represents the main abiotic limitation affecting the production (Chaves and Oliveira, 2004). A component of drought tolerance is the production and accumulation of osmotically active substances, known as osmotic adjustment (Carvalho, 2005). Several plants modify their metabolism under water deficit by accumulating organic solutes such as organic acids, soluble carbohydrates, amino acids, and proline (Lobato et al., 2008b), that will act in plant osmotic adjustment (Azevedo Neto, 2005; Mittler, 2006).

Proline is an amino acid synthetised from glutamate or arginine under normal conditions, with the glutamate route being preferential during water deficit (Pulz, 2007). The synthesis of this amino acid has an important role in plants exposed to water deficit, because it is strongly related to maintenance of water potential in plant tissues (Kerbauy, 2004). Plants under conditions of water deficit or salt stress contain higher levels of proline (Teixeira and Pereira, 2007).

The glycinebetaine is synthetised from choline or glycine (Meneses et al., 2006), as this amino acid is extensively distributed throughout the plant during stress conditions. Normally an over-production of glycinebetaine occurs, resulting in negative interferences provoked by change in environment (Ashraf and Foolad, 2007).

Nitrogen assimilation is related to the photosynthesis process and carbon metabolism due to necessity of energy to carry metabolic reactions with ATP (Adenosine triphosphate) consumption, and also carbon supply during amino acids formation like proline and glycinebetaine (Bredemeier and Mundstock, 2000).

Recently, benefits of silicon application has been reported on physiological parameters such as transpiration (Lobato et al., 2009a), stomatal conductance (Gunes et al., 2007), and photosynthesis (Sacala, 2009), which probably will influence nitrogen metabolism indirectly. Therefore, it is necessary to investigate silicon action, and optimal concentration to be used in this culture.

The osmotic adjustment is a well-described characteristic in many drought tolerant plants species (Trovato et al., 2008; Oliveira Neto et al., 2009), which is usually triggered by proline and/or glycinebetaine accumulation (Sankar et al., 2007; Parida et al., 2008; Lobato et al., 2009b). However, there is limited information linked to nitrogen compounds responsible for osmotic adjustment in *Capsicum annuum* plants.

This study aims to (i) investigate the silicon action on nitrogen metabolism and (ii) elucidate the mechanisms responsible for the osmotic adjustment in two *Capsicum annuum* cultivars exposed to water deficiency.

Results

Silicon attenuation on leaf relative water content, stomatal conductance, and transpiration rate

The water deficit promoted a decrease in leaf relative water content in two cultivars, and 0.25, 1.00, and 1.75 µM Si did not consistently increase this variable in Ikeda, although it was maintained at levels closer to the deficit. In all Si concentrations the leaf relative water content was slightly higher than the deficit + 0 µM Si in Vermelho Gigante (Fig 1 A).

Stomatal conductance was significantly reduced due to water deficit in both cultivars, compared to control. The Ikeda cv. showed higher stomatal conductance under 1.00 and 1.75 µM Si compared to deficit + 0 µM Si, while Vermelho Gigante had higher values at 0.25 and 1.75 µM Si (Fig 1B).

The water deficit caused significant reduction in transpiration in Ikeda and Vermelho Gigante cultivars (Fig 1C). Exogenous application of 0.25, 1.00, and 1.75 µM Si promoted attenuation of symptoms induced by water deficit. The treatments with added silicon were not statistically different.

Silicon action on nitrate reductase activity and free ammonium

The nitrate reductase activity in Ikeda and Vermelho Gigante cultivars was significantly reduced with water deficit (Fig 2 A), compared with control treatment. Si at 0.25, 1.00, and 1.75 µM did not increase nitrate reductase activity in Ikeda cultivar, compared to deficit + 0 µM Si. Vermelho Gigante had similar nitrate reductase activity at 1.75µM Si, compared to control. Based on this variable, the optimal Si concentration is 1.75 µM for both Ikeda and Vermelho Gigante. Correlation analysis revealed a positive and significant relationship between leaf relative water content and nitrate reductase activity ($r = 0.85$; $P < 0.01$) (Fig 3).

Concentrations of free ammonium in two cultivars were not significantly different in treatments under water deficit and progressive concentrations of Si compared with the control. However, Vermelho Gigante had higher free ammonium compared with Ikeda for all treatments (Fig 2 B). In this parameter, the silicon application had no significant effect.

Silicon interference linked to total soluble amino acids and total soluble proteins

Water deficiency caused a significant increase in total soluble amino acids in Ikeda, compared to the control (Fig 4 A). The concentration of 0.25 µM Si increased total soluble amino acid in Ikeda compared to control and 0 µM Si. However, all Si concentrations did not show any differences between treatments in Vermelho Gigante. The concentration of total soluble proteins under water deficit was not different compared with control. Si applications did not significantly increase total soluble proteins compared to control and deficit + 0 µM Si (Fig 4 B).

Changes in proline and glycinebetaine

The water deficiency increased proline in both cultivars, being significant only in Ikeda. Proline in Ikeda for 0.25 and 1.00 µM Si were not different from deficit + 0 µM Si, but were lower in 1.75 µM Si. For Vermelho Gigante, proline levels were higher with 0.25 and 1.75 µM Si compared to control and deficit + 0 µM Si (Fig 5 A).

Glycinebetaine levels were slightly increased due to water deficit (Fig 5 B), compared with the control in both cultivars. Higher glycinebetaine concentration was found in Ikeda at 0.25 µM Si compared to control and deficit + 0 µM Si. In Vermelho Gigante, there were no significant differences in glycinebetaine for all Si concentrations compared to control. The correlation analysis demonstrated a linear and negative relationship between glycinebetaine and free ammonium ($r = -0.91$; $P < 0.01$) (Fig. 6).

Discussion

The leaf relative water content of treatments under silicon application was maintained at levels closer to the control treatment. This can be linked to silicon action that is probably absorbed by plant, and deposited mainly in epidermal cell walls (Savant et al., 1997). Additionally, the Si can contribute to higher resistance of xylem vessels (Ma et al., 2004), which are structures responsible by water transport into plant (McElrone et al., 2004). Therefore, plants with firmer xylem vessel walls can potentially avoid problems in these structures during drought or extreme heat, besides increasing water volume assimilated by plants (Sperry et al., 2002). Romero-Aranda et al. (2006) investigated silicon effects on

Lycopersicon esculentum plants under salt stress. Their data was in agreement with ours in this investigation.

The Si application attenuated the effects of water deficit, indicating intermediary levels in relation to stomatal conductance, and consequently, possible maintenance in gas exchange. A fall in this parameter will affect directly water relations, limiting the assimilation of carbon dioxide (CO_2) and water flux (H_2O) through stomata (McDermit, 1990). The stomatal mechanism will reduce the CO_2 assimilation, causing a reduction in photo-assimilate production and losses in yield (Paiva et al., 2005). Similar results were observed by Gong et al. (2005) which showed the silicon effects on *Triticum aestivum* plants under water deficit, with stomatal conductance being kept at intermediary levels in relation to control plants.

Silicon promoted attenuation in symptoms linked to water deficiency in transpiration rate, because plants absorbed the silicon in form of monosilicic acid (H_4SiO_4) (Richmond and Sussman, 2003) and silicon accumulates in the leaf, forming a doubled layer. This accumulation promotes a reduction in transpiration and decrease water loss by the plant (Freitas et al., 2011; Datnof et al., 2001), but still contributes to the maintenance of adequate transpiration rate. In addition, the transpiration process in plants is carried out by stomata in leaf and normally cuticles in stem (Kerbauy, 2004). Agarie et al. (1998) found improvement linked to transpiration rates in *Oryza sativa* plants cultivated under Si presence. Similar results on maintenance of transpiration were reported previously by Lobato et al. (2009a) who studyied the protective action of silicon in *Capsicum annuum* under water deficit.

The silicon treatments slightly increased nitrate reductase activity, and this is probably indirectly linked to maintenance of transpiratory flux. As transpiration occurs mainly by stomata and partially by cuticle (Kerbauy, 2004), the silicon attenuates the negative symptoms induced by water deficit keeping stomata opened, and consequently maintaining the transpiration rate. Reduction of this parameter in water stressed plants occurs normally due to reduction in water absorption. As a consequence, any enzymes such as nitrate reductase (Meyers Júnior et al., 1986) will be inhibited due to the limitation in water supply. This is related to a reduction in leaf relative water content (Fig 3). Nitrate reductase is the first enzyme in route of nitrate assimilation, and represents a limiting step in incorporation of this nutrient (Campbell, 1988). Hence, it has been frequently utilised as a stress indicator and other changes associated to modulator factors of plant growth (Srivastava, 1980; Carelli et al., 1996). Similar results were reported by Lobato et al. (2009c) on *Glycine max*.

Similar free ammonium levels suggest that Si application does not change this parameter under water deficit. In nitrogen metabolism, the route of ammonium assimilation is normally secondary, with the main route mediated by nitrate reductase (NR), with reduction from nitrate (NO_3^-) to nitrite (NO_2^-), and by nitrite reductase (RiN) action for the conversion of nitrite in ammonium (NH_4^+). The ammonium is assimilated and transported to form amino acids like as glutamine and glutamate, which work by translocating organic nitrogen from source to sink (Ferreira et al., 2002). In addition, this investigation reveals a negative relationship between free ammonium and glycinebetaine, suggesting that the pathway used by organic nitrogen to from glycinebetaine is the ammonium route.

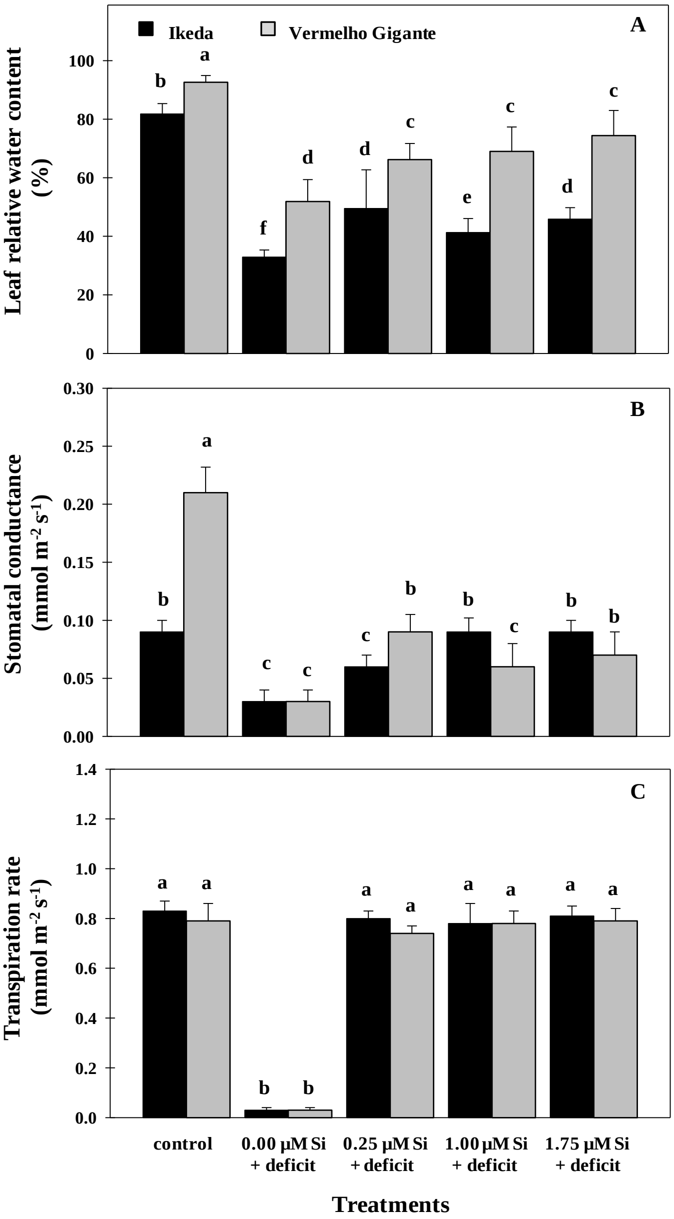

Fig 1. Leaf relative water content (A), stomatal conductance (B), and transpiration rate (C) in two pepper cultivars treated with silicon and exposed to water deficiency. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% probability. The bars represent the mean standard error.

Data reported in this study corroborate the observations of Lobato et al. (2009d) studying *Capsicum annuum* plants.

The increase in amino acids levels in Ikeda cultivar are connected to maintenance of free ammonium combined with higher nitrate reductase activity in this cultivar. The incorporation of ammonium is carried out by enzymes glutamine synthetase (GS) and synthase glutamate (GOGAT) (King et al., 1993; Crawford, 1995) resulting in amino acid formation. Then, these amino acids will be distributed to organs as leaf, stem and root due to low synthesis rate of amino acids under water deficiency condition (Taiz and Zeiger, 2004). In addition, several species have higher affinity for nitrate which is absorbed more quickly than ammonium, while others have a preference for ammonium.

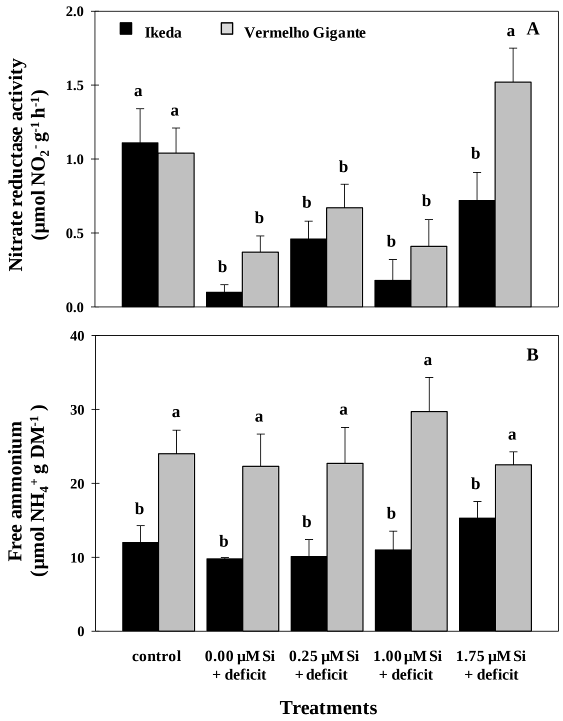

Fig 2. Nitrate reductase activity (A) and free ammonium (B) in two pepper cultivars treated with silicon and exposed to water deficiency. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

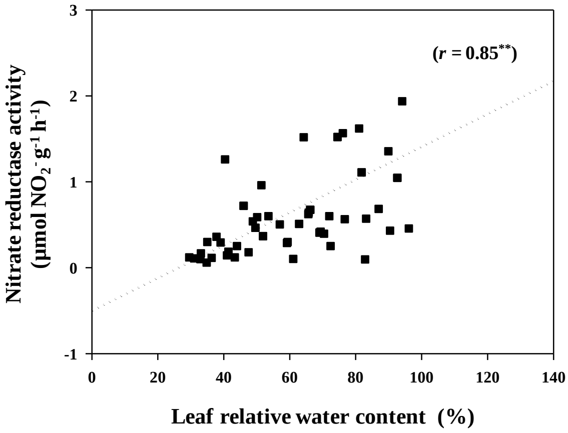

Fig 3. Relationship between leaf relative water content and nitrate reductase activity in two pepper cultivars treated with silicon and exposed to water deficiency. Asterisks (**) indicate significance at 0.01 probability level.

Therefore, in *Capsicum annuun* the ammonium ion is probably the main form of nitrogen absorbed during water deficit condition (Cruz et al., 2006). Experiments revealed that nitrogen efficient genotypes have a high capacity for incorporation of ammonium ion in amino acids through GS and GOGAT enzymes (Machado et al., 1992). Similar results were found by Lobato et al. (2008b) working with *Glycine max*.

Our data indicate that water stress did not provoke a change in total soluble proteins. Our results also contrast with those reported by Zhu et al. (2004) studying *Cucumis sativus* under salt stress and silicon application. Situations of water deficit can stimulate the synthesis of several protective proteins such as LEA (late embryogenesis abundant) and chaperones (Zhu, 2001). Mansour (2000) related the accumulation of soluble proteins during water stress to the reserve of nitrogen for use after the stress situation.

Silicon treatments increased proline in both cultivars, and this is possibly related to the secondary effects produced by silicon action on transpiration because the Si kept transpiratory flux at similar levels close to control plants. This amino acid was probably not generated by protease enzymes or due to a change in free ammonium and proteins levels. The increase in proline in plants causes a higher affinity for water (H_2O) under water deficit, and based on this strategy, increase water retention in tissue, which consequently increase plant tolerance to water deficit (Lobato et al., 2009d). Lobato et al. (2009b) confirmed the data of this investigation on *Capsicum annuum* subjected to silicon action and water deficiency.

Our data suggests that glycinebetaine also works as an osmoregulator in *Capsicum annuum* cultivars. The glycinebetaine is an amino acid responsible for osmo-regulation in several species, acting as organic solute. Its accumulation does not increase energy consumption or interfere in plant metabolic activity (Lobato et al., 2009b). Sankar et al. (2007) studied the glycinebetaine in five *Abelmoschus esculentus* varieties exposed to water restriction and found the similar results.

Materials and methods

Experimental conditions

The study was carried out in the Instituto de Ciencias Agrárias (ICA) of the Universidade Federal Rural da Amazonia (UFRA), Belem city, Para state, Brazil (01°27'S and 48°26'W). Plants remained in the greenhouse without environment control. The minimum, maximum and medium temperatures were 22.1, 35.5, and 28.4°C, respectively. Air relative humidity during experimental period oscillated between 65 and 93%. Photoperiod was 12 h of light.

Plant material, substrate and pot

In this study, we used seeds of pepper (*Capsicum annuum* L.) cvs. Ikeda and Vermelho Gigante, which have high commercial values. Substrate to plant growth was composed mixture of sand and silic in proportion of 2:1, respectively. This substrate was autoclaved at 120°C atm^{-1} for 40 min. The container used was pot type Leonard with 2 L capacity.

Experimental design and treatments

Experimental design was a completely randomised factorial layout composed of five water and silicon combinations (control, deficit + 0.00, deficit + 0.25, deficit + 1.00, and deficit + 1.75 µM Si) applied to two cultivars (Ikeda and Vermelho Gigante) with a total of 10 treatments. Experiment was assembled with six replicates and 60 experimental units, as well as one plant in each unit.

Plant culture and silicon application

Five seeds were placed in each pot, and thinned to one plant per pot after germination. Control and deficit + 0.00 µM Si

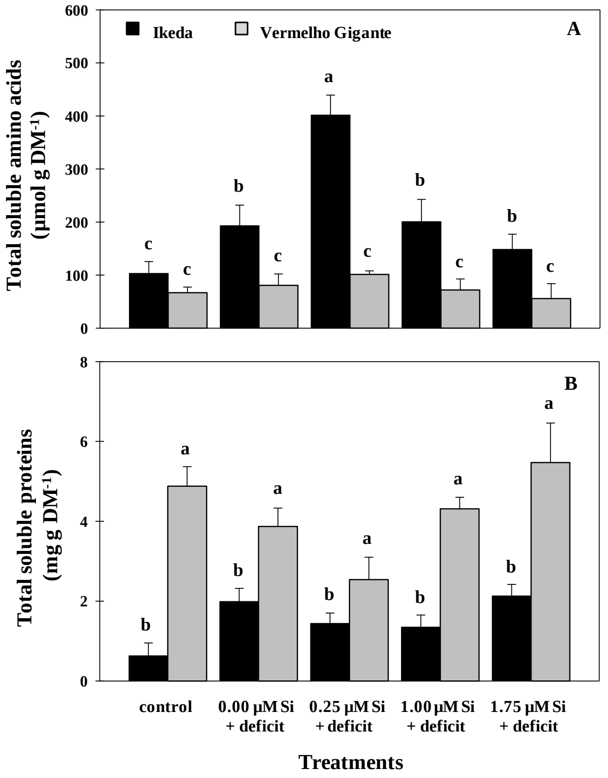

Fig 4. Total soluble amino acids (A) and total soluble proteins (B) in two pepper cultivars treated with silicon exposed to water deficiency. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

treatments received macro and micro nutrients in the form of nutrient solution of Schwarz (1995), without Silicon (Si). Treatments deficit + 0.25, deficit + 1.00, and deficit + 1.75 µM Si received the same Schwarz (1995) nutrient solution, with addition of silicon through sodium metasilicate ($Na_2SiO_39H_2O$), in agreement with Liang et al. (2006) and adapted in Laboratorio de Fisiologia Avancada (LFVA). The solutions were applied in plants for a period of 45 days, and the nutrient solutions were changed every 5 days at 09:00 h and the pH of the nutrient solution was adjusted to 6.0±0.1 with addition of HCl or NaOH. One the 45th day after the experiment implementation, plants of the treatments under deficit + 0.00, deficit + 0.25, deficit + 1.00, and deficit + 1.75 µM Si were subjected to a period of 6 days without nutrient solution (Lobato et al., 2009b), in which the water deficit was simulated from the 65th until 71th day after the experiment started. After this period, physiological parameters were measured in these plants.

Leaf relative water content

Leaf relative water content was evaluated with 40 leaf disks with 10 mm diameter being removed from each plant, and the calculation was done according to the formula proposed by Slavick (1979): $LRWC = [(FM – DM)/(TM – DM)] \times 100$
Where; FM is fresh matter, TM is turgid matter evaluated after 24 h and saturation in deionised water at 4°C in dark and DM is the dry matter determined after 48 h in oven with forced air circulation at 80°C.

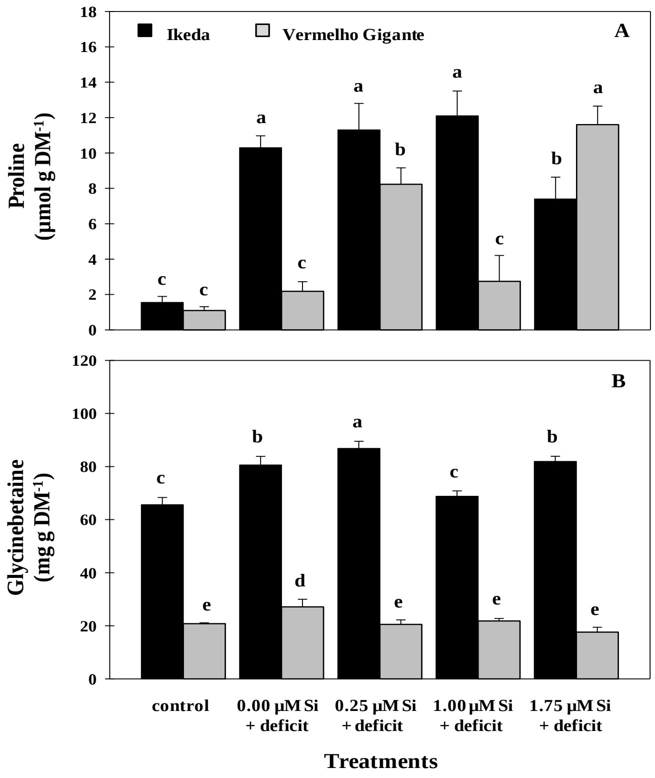

Fig 5. Proline (A) and glycinebetaine (B) in two pepper cultivars treated with silicon exposed to water deficiency. Means followed by the same letter are not significantly different by the Scott-Knott test at 5% of probability. The bars represent the mean standard error.

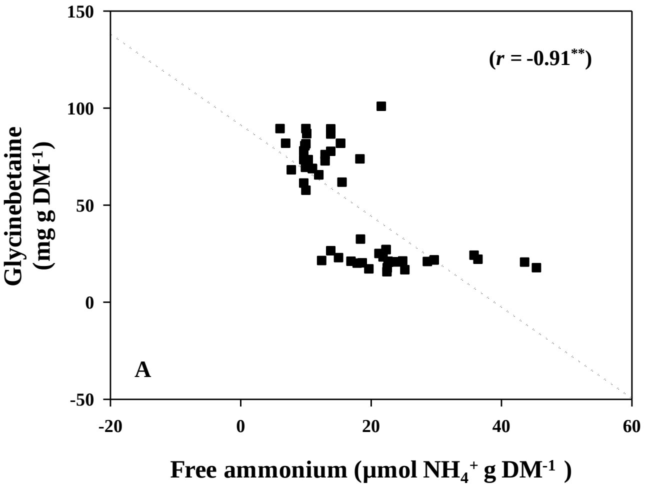

Fig 6. Relationships between free ammonium and glycinebetaine in two pepper cultivars treated with silicon and exposed to water deficiency. Asterisks (**) indicate significance at 0.01 probability level.

Water relations

Stomatal conductance and transpiration were evaluated in fully expanded leaves located in the medium third of the branch main under light, using a steady state porometer (LI-COR Biosciences, model 1600), with the gas changes evaluated immediately during the period between 10:00 and 12:00 h in all the plants.

Harvest and tissue preparation

Plants were harvested and divided into shoot and root, with cut located in plant collar. Subsequently, fresh tissues were used for nitrate reductase activity.

In vivo nitrate reductase activity

Nitrate reductase enzyme (E.C. 1.6.6.1) was extracted from leaf and root until a weight of 200 mg was reached and the samples were incubated in 5 mL of extraction buffer (KH_2PO_4 at 0.1M, KNO_3 at 50 mM, isopropanol at 1% (v/v) and pH 7.5) for 30 minutes at 30ºC. All the procedures carried out in the dark. The quantification of the enzyme activity was conducted according to the method of Hageman and Hucklesby (1971) using a spectrophotometer with absorbance at 540 nm (Quimis, model Q798DP).

Dehydration and sample preparation

Leaf and root were harvested and placed in an oven with forced air circulation at 70 ± 2ºC for 96 h. After this period, shoot and root dry matter were triturated, with the resulting powder kept in glass containers. These containers were stored in the dark at 15ºC for nutritional and biochemical analysis later on.

Free ammonium

Fifty mg of leaf dry matter powder was incubated in 5 ml of sterile distilled water at 100°C for 30 min. The homogenised mixture was centrifuged at 2.000 *g* for 5 min at 20°C and the supernatant was removed. The quantification of the free ammonium was carried out at 625 nm in agreement with Weatherburn (1967), and $(NH_4)_2SO_4$ (Sigma Chemical) was used as standard.

Total soluble amino acids

Fifty mg of leaf dry matter powder was incubated with 5 mL of sterile distilled water at 100ºC by 30 minutes and the homogenised mixture was centrifuged at 2.000 *g* for 5 minutes at 20ºC and supernatant was removed. Quantification of the total soluble amino acids was carried out at 570 nm according to Peoples et al. (1989), using L-asparagine + L-glutamine (Sigma Chemicals) as standard.

Total soluble proteins

Determination of the total soluble proteins was carried out with 100 mg of powder, incubated in 5 mL of extraction buffer (Tris-HCl at 25 mM and pH 7.6) then omogenised mixture was kept in agitation by 2 h, and centrifuged at 2.000 *g* for 10 minutes at 20ºC. Quantification of the total soluble proteins was carried out at 595 nm in agreement with Bradford (1976), using albumin bovine (Sigma Chemicals) as standard.

Data analysis

Data were submitted to variance analysis and when significant differences occurred, the Scott-Knott test at 5% level of error probability was applied. Standard errors were calculated for all means. The correlation analysis was performed using the Pearson parametric method (Steel et al., 2006), and statistical analyses were carried out with the SAS software (SAS Institute, 1996).

Conclusion

Data obtained in this study revealed positive inference of silicon on water relations and nitrogen compounds, as well as an improvement in osmotic adjustment in *Capsicum annuum* plants exposed to water deficit. Proline and glycinebetaine contributed to osmotic adjustment in Ikeda, while this process in Vermelho Gigante is carried only by glycinebetaine.

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